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Award Number: W81XWH-10-1-0985

TITLE: Structural Rearrangements in DNA Repair Genes in Breast Cancer

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REPORT DATE: October 2011

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*
01-10-2011

2. REPORT TYPE
Annual

3. DATES COVERED *(From - To)*
20 Sep 2010 - 19 Sep 2011

4. TITLE AND SUBTITLE
Structural Rearrangements in DNA Repair Genes in Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0985

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Pittsburgh
Pittsburgh, PA 15213

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The genetic basis of cancer has been firmly established in the last few decades. Genomic instability is a hallmark feature of virtually all breast cancer cells, and is caused either by inherited mutations in genes that control genomic fidelity and stability (particularly in DNA repair pathways), or somatic mutations that are acquired during breast cancer progression. We hypothesized that structural genomic alterations in the genes that are actually themselves involved in DNA repair enhance the level of genomic instability and ultimately affect breast cancer progression and prognosis. We cloned a fusion gene of RAD51C:ATXN7 which exists in two spice isoforms. We found the same fusion in two other breast cancer cell lines and a prostate cancer cell line. We have transfected MCF10A and MCF-7 cells with a GFP reporter of DNA repair activity and are testing the effect of this mutation on DNA repair. We are also using COMET assays. We have also identified multiple truncations of the EYA family. We are expanding these studies and understanding the biological relevance of the findings.

15. SUBJECT TERMS
No subject terms provided.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	9	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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1) Introduction

The genetic basis of cancer has been firmly established in the last few decades. Genomic instability is a hallmark feature of virtually all breast cancer cells, and is caused either by inherited mutations in genes that control genomic fidelity and stability (particularly in DNA repair pathways), or somatic mutations that are acquired during breast cancer progression. The importance of DNA repair in breast cancer is highlighted by the fact that inherited breast cancer is associated with germline mutations in ten different genes associated with genome stability and fidelity. Importantly, the central role of DNA double stranded break repair (DSBR) in both hereditary and sporadic breast cancer may provide an Achilles heel that can be targeted therapeutically. Thus, defects in DSBR pathways lead cells to become hypersensitive to DNA damaging agents such as mitomycin C or cisplatin. Using paired end sequencing, we generated a map of breaks in genomic DNA in a breast cancer cell line named MCF-7. This study gave us a unique insight into the genomic instability in MCF-7 cells and showed that a number of genes that had undergone structural change (translocation, deletion, or inversion) were tumor suppressor genes and were mostly repaired by non-homologous end joining an error-prone method of DNA double strand break repair. Intriguingly, we identified translocation of three genes, *RAD51C*, *BRIP1*, and *EYA2*, all of which are all central to DSBR, leading to the novel and exciting IDEA that genes important for genomic integrity and homologous recombination are themselves structurally altered at the genomic level and thus potentially non-functional.

We hypothesize that structural genomic alterations in the genes that are actually themselves involved in DNA repair enhance the level of genomic instability and ultimately affect breast cancer progression and prognosis. We hypothesize that alterations in *BRIP1*, *RAD51C, and EYA2* will render cell hypersensitive to DNA damaging agents and that fidelity of the DSBR pathway, measured at the genomic level, may be a candidate biomarker for personalizing therapy.

SPECIFIC AIMS

1) Determine the prevalence of recurrent and selected aberrations in BRIP1, RAD51C, and EYA2 in a large cohort of human breast tumors, and correlate presence with prognosis and/or response to therapy.

2) Test whether truncations or fusions of BRIP1, RAD51C and EYA2 result in loss of function or dominant negative effects on DNA repair, sensitization to DNA damaging agents, and if the loss of these proteins contributes to genomic instability.

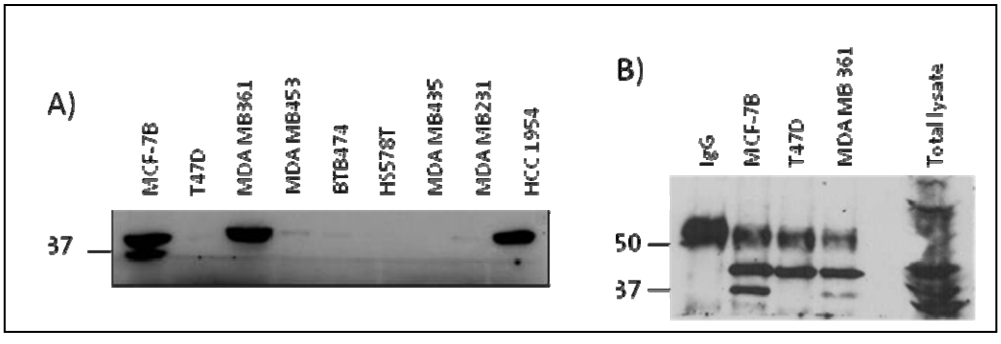
2) Body

I would like to note that this grant received notification of funding just as I was leaving Baylor College of Medicine and being recruited to the University of Pittsburgh Cancer Institute. The grant started two months after my arrival, and at a time when I was in temporary laboratory space and in the process of hiring personnel. Since that time my laboratory has moved into its permanent space (April 2011) and I have hired personnel. We have since made progress on several of the sub-aims but are slightly behind scheduled due to the downtime of the move.

Aim 1

1) Analyze BRIP1, RAD51C, and EYA2 protein in 20 breast cancer cells lines by immunoblot (months1-4)

We measured RAD51C in a range of breast cancer cell lines. We noted varying levels with high expression in MCF-7, MDAMB361 and HCC1594 (Figure 1A). However, based upon the translocation we had observed and the predicted truncated product it would have generated we were particularly looking for small isoforms of RAD51C. MCF-7 showed a prominent truncated product, and immunoprecipitation followed by immunoblotting revealed that MDAMB361 had the same



product (Figure 1B). This truncated isoforms was further verified by immunoprecipitation with one RAD51C antibody followed by immunoblotting with a different RAD51C antibody to confirm specificity (data not shown).

However, as noted in the next subaim, measurement of RAD51C mRNA has now shown that the major isoforms of a RAD51C:ATXN7 fusion is actually a full length splice isoforms that is predicted to actually be a much higher molecular weight than wild-type RAD51C. As we were not initially searching for this size we didn't consider this and thus need to repeat the immunoblotting.

We have also immunoblotted for BRIP1 and found highly variable levels between cell lines, perhaps indicative of loss of one allele in some cell lines (data not shown). We have not found suitable EYA2 antibodies for immunoblotting.

2) Measure BRIP1, RAD51C, and EYA2 mRNA by RT-PCR (months 1-4).

As my laboratory has moved and changed personnel, we first confirmed that the BRIP1, RAD51C, and EYA2 DNA translocations were present in different batches of MCF-7 cells (and not in the non-tumorigenic cell line MCF10A) (Figure 2). As noted, PCR of these translocations showed them all to be present.

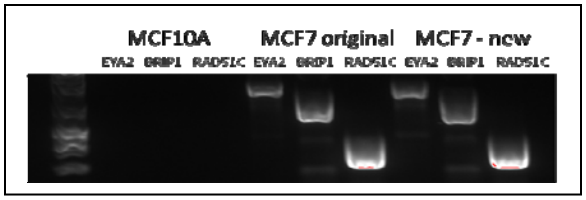


Figure 2: DNA PCR of BRIP1, RAD51C, and EYA2 translocations in two different batches of MCF7, but not MCF10A cells.

We next ran RT-PCR of RAD51C-ATXN7 to examine and clone mRNA isoforms. We isolated the original splice isoforms which spliced exon 7 of Rad51C to ATXN7 we reported in the original manuscript (1). This resulted in an early translation stop codon and a truncated RAD51C protein (Figure 3A - schematic; 3B – sequence). However, we now also cloned a different splice variant which fused exon 6 of RAD51C to exon 6 of ATXN7 and resulted in a complete in frame transcript (Figure 3A - schematic; 3C – sequence). This is obviously exciting as this may produce a novel gene fusion that expresses the ATXN7 in an aberrant fashion.

Figure 3: Identificaiton of short and full-length RAD51C-ATXN7 fusion mRNAs. A) Schematic of the short and long RAD51C:ATXN7 fusions. In the short isoforms, an aberrant splice causes a premature translation stop codon. The long isoform is in frame and contains all exons. B) Raw sequence of the expressed and cloned short form mRNA. Note that the fusion (splicing exon 7) is shorter than wild-type RAD51C. C) Raw sequence of the expressed and cloned long form mRNA. Note that the fusion (splicing exon 6) is much longer than wild-type RAD51C. The excess sequence is ATXN7.

We used primer specific RT-PCR to estimate levels of these fusion genes and found that the full length fusion is the abundant mRNA (data not shown).

This has important implications for our work as it suggests that the fusion protein will be larger than wild-type RAD51C (affecting #1 above) and also suggests that the ATXN may have some function.

We screened other breast cancer cell lines for the RAD51C:ATXN7 fusion mRNA and found it in several breast cancer cell lines (Figure 5) and LAPC4 prostate cancer cell line.

We have yet to measure mRNA levels of BRIP1 or EYA2

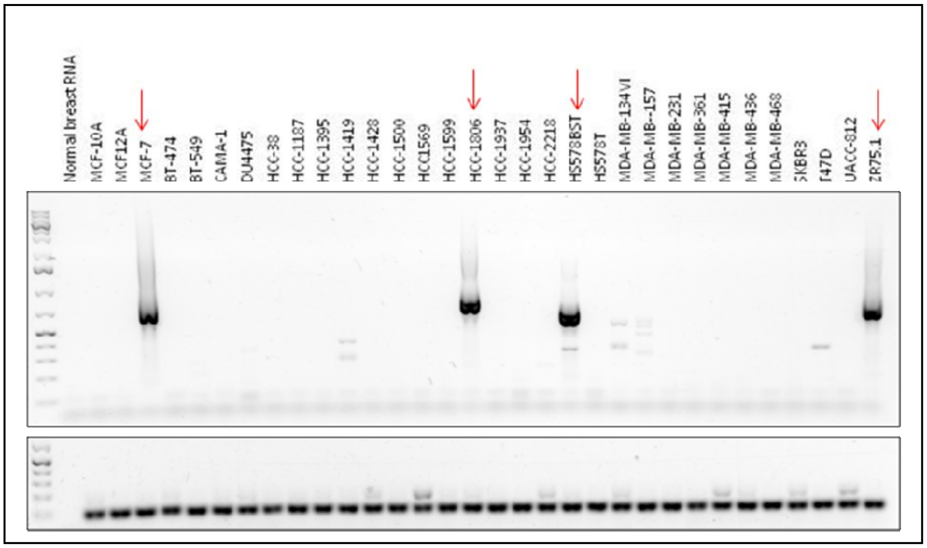


Figure 4: RT-PCR using 5' RAD51C and 3' ATXN7 primers in a panel of breast cancer cell lines. MCF7 serves as a positive control for the fusion. Note the same RT-PCR product in HCC1906, HS578T, and ZR75.1. Disappointingly, we didn't see the fusion in MDAMB361, where we previously found a truncated RAD51C protein product (Figure 1) – we are currently investigating why this is.

3) *Measure specific translocations in BRIP1, RAD51C, and EYA2 using PAMP in 20 cell lines. Novel translocations will be identified using Alu or targeted gene walking PCR (months 4-12).*

Measurement of translocations is complicated by the fact that one doesn't necessarily know the identity of the fusion partner, leading to complicated time consuming methods such as PAMP and gene walking PCR. We tried gene walking PCR but with no success. To obviate this, we have recently collaborated with Tom Walsh Ph.D. at Washington University who has recently used capture-sequencing to analyze BRCA1 mutations. Tom is now using this technology to measure changes in RAD51C, and we have sent him MCF-7 genomic DNA, and the exact identify of our translocation as a positive control. Unfortunately, the first round of capture sequence provided data that was suboptimal with too few sequence reads and this is now being repeated.

4) Analyze specific translocations in BRIP1, RAD51C, and EYA2 identified in the cell lines in a pilot study of human breast tumors (n=50) and then a larger definitive set of 200 (months 6-18).

Not yet started

5) Measure specific translocations in BRIP1, RAD51C, and EYA2 in a pilot study of 50 breast tumors using break-away fish, and then a larger definitive studying across stages of breast cancer progression (months 12-18).

Not yet started.

Aim 2

1) Analyze localization of translocations identified in Aim 1 using fluorescent microscopy of GFP-tagged proteins (months 12-18).

We cloned a myc tag onto the short and long isoforms of RAD51C:ATXN7 to examne expression and localization of the fusion products. We are currently expressing them and will detect their localization.

2) Test whether the translocation products fail to localize to sites of DNA damage (highlighted by H2AX and RAD51 foci) following irradiation (months 12-18)

Not yet started

3) Test whether expression of the translocations, or a reduction in expression of BRIP1, RAD51C, and EYA2 causes reduced double stranded break repair activity (months 14-20) .

To start this aim we have set up two types of assays for measuring double stranded break repair activity. The first is the COMET assay, which is the gold standard. We will do this in cells treated with chemotherapy or with radiation. We have also transfected MCF10A and MCF-7 cells with a GFP reporter which is a reported of DNA repair activity. The method is shown in Figure 6A. We have stably transfected MCF-10A cells with the GFP-reporter plasmid and isolated stable clones which express this plasmid (Figure 6B). We will now express the fusion genes and examine their effect on DNA repair.

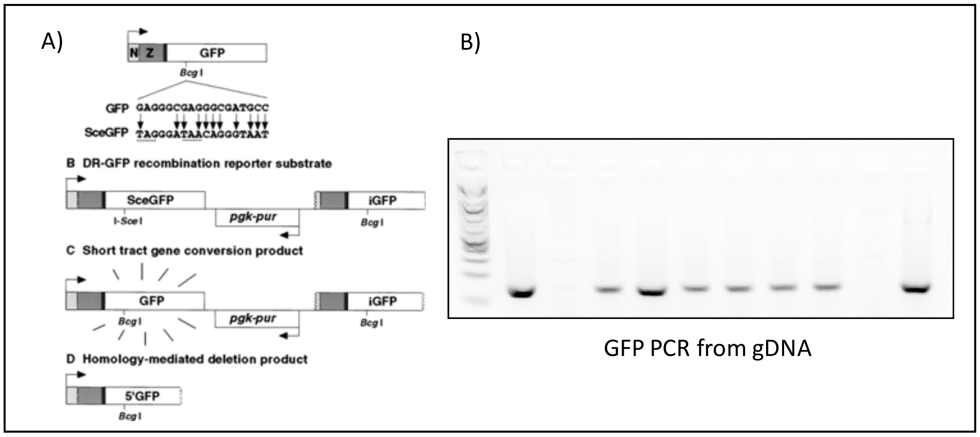


Figure 5: Assay for DNA repair in MCF10A cells: A) Schematic of the GFP reporter assay for measuring DNA repair. B) Stable esxpression of the GFP reporter plasmid in MCF10A stable clones.

4) Test the effect of the translocations and/or a reduction in expression of BRIP1, RAD51C, and EYA2 on response to DNA damaging agents and other cell biological responses in normal, immortalized and breast cancer cell lines (months 14-24).

Not yet started, but will use assays developed above.

3) Key Research Accomplishments

- MCF-7 cells have a genomic translocation of RAD51C and ATXN7 resulting in the generation of two different mRNA splice isoforms, one containing the N terminus of RAD51C and the full C-terminus of ATXN7
- RAD51C:ATXN7 mRNA fusion exists in several breast cancer cell lines including MCF-7, HCC1906, HS578T, and ZR75.1. The same fusion has been detected in MDAMB361, although this has been difficult to reproduce

4) Reportable Outcomes

Adrian V. Lee, Petra den Hollander, Oliver A. Hampton, Cristian Coarfa, Aleksandar Milosavljevic. Structural rearrangements in DNA repair genes in human breast cancer. DOD Era of Hope Meeting, Aug 2011, Florida**.**

5) Conclusion

The proposal was initially delayed by my move from Baylor College of Medicine to the University of Pittsburgh. However, in the first year of this proposal we have started the majority of the subaims. We have identified and cloned a novel full length RAD51C:ATXN7 mRNA isoforms and found the same mRNA in other breast cancer cell lines. We spent time setting up numerous DNA repair assays which will be valuable in YR2. We have identified a long splice isoforms of a fusion gene RAD51C:ATXN7 and will investigate the function of this gene. With Tom Walsh we will use capture-seq to identify novel fusion genes in DNA repair genes. This area of research continues to be of very high intrigue and we expect the next year will provide mechanistic insight into the biology of these fusion genes and provide a rationale for this further study.

6) References

1) Hampton OA, Den Hollander P, Miller CA, Delgado DA, Li J, Coarfa C, Harris RA, Richards S, Scherer SE, Muzny DM, Gibbs RA, Lee AV, Milosavljevic A. A sequence-level map of chromosomal breakpoints in the MCF-7 breast cancer cell line yields insights into the evolution of a cancer genome. Genome Res. 2009 Feb;19(2):167-77.

7) Appendix – Meeting Abstract

Structural rearrangements in DNA repair genes in human breast cancer.

Adrian V. Lee, Petra den Hollander, Oliver A. Hampton, Cristian Coarfa, Aleksandar Milosavljevic.

DOD Era of Hope Meeting, Aug 2011, Florida**.**

The genetic basis of cancer has been firmly established in the last few decades. Genomic instability is a hallmark feature of virtually all breast cancer cells and is caused either by inherited mutations in genes that control genomic fidelity and stability (particularly in DNA repair pathways) or somatic mutations that are acquired during breast cancer progression. The importance of DNA repair in breast cancer is highlighted by the fact that inherited breast cancer is associated with germline mutations in ten different genes associated with genome stability and fidelity. Importantly, the central role of DNA double-strand break repair (DSBR) in both hereditary and sporadic breast cancer may provide an Achilles heel that can be targeted therapeutically. Using new and innovative sequencing methods, we generated a map of breaks in genomic DNA in a breast cancer cell line named MCF-7. This study gave us a unique insight into the genomic instability in MCF-7 cells and showed that a number of genes that had undergone structural change (translocation, deletion, or inversion) were tumor suppressor genes and were mostly repaired by nonhomologous end joining, an error-prone method of DNA DSBR. Intriguingly, we identified translocation of three genes, RAD51C, BRIP1, and EYA2, all of which are all central to DSBR, leading to the novel and exciting IDEA that genes important for genomic integrity and homologous recombination are themselves structurally altered at the genomic level and thus potentially nonfunctional. The RAD51C translocation results in a fusion gene (RAD51C:ATXN7), which was subsequently found expressed in two more breast cancer cell lines. We hypothesize that structural genomic alterations in the genes that are actually themselves involved in DNA repair enhance the level of genomic instability and ultimately affect breast cancer progression and prognosis. Ongoing work is examining the prevalence of recurrent and selected aberrations in BRIP1, RAD51C, and EYA2 in breast cancer cell lines and primary tumors, and we will then test whether truncations or fusions of BRIP1, RAD51C, and EYA2 result in loss of function or dominant negative effects on DNA repair, sensitization to DNA damaging agents, and if the loss of these proteins contributes to genomic instability.